



Research article

Disentangling the effect of atmospheric CO₂ enrichment on the halophyte *Salicornia ramosissima* J. Woods physiological performance under optimal and suboptimal saline conditions



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ABSTRACT

A mesocosm experiment was designed to assess the effect of atmospheric CO₂ increment on the salinity tolerance of the C₃ halophyte *Salicornia ramosissima*. Thus, the combined effect of 400 ppm and 700 ppm CO₂ at 0, 171 and 510 mM NaCl on plants growth, gas exchange, chlorophyll fluorescence parameters, pigments profiles, anti-oxidative enzyme activities and water relations was studied. Our results highlighted a positive effect of atmospheric CO₂ increment on plant physiological performance under suboptimal salinity concentration (510 mM NaCl). Thus, we recorded higher net photosynthetic rate (A_N) values under saline conditions and 700 ppm CO₂, being this effect mainly mediated by a reduction of mesophyll (g_m) and biochemical limitation imposed to salt excess. In addition, rising atmospheric CO₂ led to a better plant water balance, linked with a reduction of stomatal conductance (g_s) and an overall increment of osmotic potential (Ψ_o) with NaCl concentration increment. In spite of these positive effects, there were no significant biomass variations between any treatments. Being this fact ascribed by the investment of the higher energy fixed for salinity stress defence mechanisms, which allowed plants to maintain more active the photochemical machinery even at high salinities, reducing the risk of ROS production, as indicated an improvement of the electron flux and a rise of the energy dissipation. Finally, the positive effect of the CO₂ was also supported by the modulation of pigments profiles (mainly zeaxanthin and violaxanthin) concentrations and anti-oxidative stress enzymes, such as superoxide dismutase (SOD) and ascorbate peroxidase (APx).

1. Introduction

Climatic change and salinization of soil are two of the greatest problems that will threat the conservation of the ecosystems worldwide (Occhipinti-Ambrogio, 2007), being soil degradation one of its main consequences (IPCC, 2007). In addition together with this conservation problem, it is expect that these environmental changes could alter the economy and health of the people (Peng et al., 2004; Naudts et al., 2013). Among the main economy activities, it seems that the productivity of agricultural systems will be severely affect by these changes (Yadav et al., 2011). Thus, climate change emerges as one of the biggest challenges for sustaining global agriculture production, being therefore crucial to find options to mitigate its effects on agricultural production.

One of such options is the use of alternative crops, species capable of growing in hostile environments (salinity and water deficit). In this regard, halophyte species have been proposed, since these plants species present tolerance mechanisms against environmental stress, high productivity and exceptional nutritional values (Ventura et al., 2015; Ventura and Sagi, 2013). Some studies have assessed the response of halophytes to NaCl salinity conditions and atmospheric CO₂ enrichment (Geissler et al. 2009a, 2009b, 2015; Hussin et al., 2017) indicating an overall improvement of photosynthetic rate and water use efficiency. However, this information is limited for a small number of species. Being necessary to increase this knowledge for a great number of species and especially for those, which are valuable resources for human activities.

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Abbreviations

RGR	Relative growth rate
RWC	Relative water content
Ψ_o	Osmotic potential
A_N	Net photosynthetic rate
g_s	Stomatal conductance
C_i	Intercellular CO ₂ concentration
iWUE	Intrinsic water use efficiency
ETR _{max}	Maximum electron transport rate
g_m	Mesophyll conductance

RuBP	Ribulose-1,5-biphosphate carboxylase/oxygenase
$V_{c,max}$	Maximum carboxylation rate allowed by ribulose-1,5-biphosphate carboxylase/oxygenase
PSII	Photosystem II
F_v/F_m	Maximum quantum efficiency of PSII photochemistry
Φ_{PSII}	PS II Operational and Maximum Quantum Yield
CAT	Catalase enzymatic activity
APX	Ascorbate peroxidase enzymatic activity
SOD	Superoxide dismutase enzymatic activity
ROS	Reactive oxygen species

Salicornia ramosissima J. Woods is a C₃ halophyte species which has been proposed as an alternative crop especially in arid and semiarid regions of the world (Lu et al., 2010). Nowadays this species is recognized as a multifunctional cash crop, since it is being used as gourmet ingredient in many countries like Spain or France along with others halophytes (Barreira et al., 2017). In addition, it possible use for feed the cattle due its edible characteristic (Isca et al., 2014) and as phytoremediation tool; (Sharma et al., 2010; Zhang et al., 2013; Pedro et al., 2013; Nunes da Silva et al., 2014; Perez-Romero et al., 2016). In addition, few species of *Salicornia* genus have demonstrated the ability to accumulate a broad variety of compounds (metabolites) with economic interest (Singh et al., 2014). However, for its efficient exploitation it is necessary to assess the evolution of this species of special interest in the near future climatic conditions. Up-to-day, the effect of the incipient rising CO₂ atmospheric concentration in this specie has not been study. Being a C₃ specie our hypothesis is that atmospheric CO₂ enrichment could lead to an increase in the photosynthetic efficiency and a better capacity to use the water with a consequent increment in biomass, as has been described for other plants species (Ghannoum et al., 2000). Despite the positives evidences, there is not a consensus about this. Since this response is specie dependent and highly influenced by others abiotic factors, such as salinity (Lenssen et al., 1993; Lenssen et al., 1995; Rozema, 1993; Geissler et al., 2009b; Geissler et al., 2010; Mateos-Naranjo et al., 2010a; b) or drought (Calvo et al., 2017). Therefore, this study was designed and conducted to: (1) determine the growth *S. ramosissima* plants in salinity treatments ranging from 0 to 510 Mm NaCl at two CO₂ concentrations (400 and 700 ppm CO₂); (2) assess the relation between the growth and photosynthetic apparatus responses, in terms of CO₂ fixation, isotopes signature PSII efficiency, photosynthetic pigments, electron transport rate energy fluxes; and (3) in the modulation of its antioxidant defence abilities.

2. Material and methods

2.1. Plant material

Seeds of *S. ramosissima*, which were collected in September 2014 from Odiel marshes (37°15'N, 6°58'O; SW Spain), were moved into a germinator (ASL Aparatos Científicos M-92004, Madrid, Spain). There, the seeds were subjected to a day-night regime of 16 h of light (photon flux rate, 400–700 nm, 35 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at 25 °C and 8 h of darkness at 12 °C, for 15 days. Then, seedlings were planted in individual plastic plots (9 cm high x 11 cm diameter) using perlite as substrate, and placed in a greenhouse with controlled conditions (temperature between 21 and 25 °C, relative humidity 40–60% and natural daylight of 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ as minimum and 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ as maximum light flux). The pots were allocated in shallow trays watering with 20% Hoagland's solution (Hoagland and Arnon, 1938) and 171 mM NaCl. Plants were kept under the previously described conditions until the experimental setup.

2.2. Experimental treatments

In October of 2015, after 3 months of seedling culture, plants with an initial height of 17 cm were randomly divided in six blocks of 10 plants. Then, they were subjected to three NaCl concentrations (0, 171 and 513 mM NaCl) in shallow trays. These salt treatments were chosen based on the concentrations that can be found in the natural distribution of the specie (Redondo-Gómez et al., 2006), being 171 mM the optimal concentration for the growth of this specie (Perez-Romero et al., 2016). The three salinities were combined with two atmospheric CO₂ concentrations (400 ppm and 700 ppm) in controlled-environment chambers (Aralab/Fitoclima 18.000 EH, Lisbon, Portugal) for 30 days. Being an annual specie, this period of time was used to avoid a decreased in the physiology variables due to age. The high level of CO₂ concentration was selected following the data from the IPCC (2014) for the next century. Chamber environmental conditions were: alternating diurnal regime of 14 h of light and 10 h of darkness, light intensity of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$, temperature between 21 and 25 °C and 40–60% relative humidity. Atmospheric CO₂ concentrations in chambers were continuously recorded by CO₂ sensors (Aralab, Lisbon, Portugal) and maintained by supplying pure CO₂ from a compressed gas cylinder (Air liquide, B50 35K).

2.3. Growth analysis

At the beginning and at the end of the experiment belowground and aboveground fractions of 10 plants for each time were separated, dried at 80 °C for 48 h and weighed. The relative growth rate (RGR) was calculated using the formula:

$$\text{RGR} = (\ln(B_f) - \ln(B_i)) D^{-1} (\text{g g}^{-1} \text{day}^{-1})$$

Where B_f = final dry mass (an average ten plants per treatment), B_i = initial dry mass (an average of the plants dried at the beginning of the experiment) and D = duration of experiment (days).

In addition, before and after the treatments, the height of the main branches and the number of branches longer than 1 cm were measured.

2.4. Water content

Relative water content (RWC) of primary branches (n = 10 per treatment) were calculated after 30 days of treatment as follow:

$$\text{RWC} = ((\text{FW} - \text{DW}) / (\text{FW} - \text{TW})) 100$$

Where FW = fresh weight of the branches, DW = dry weight after oven-drying at 80 °C for 48 h and TW = turgid weight after being in water at 4 °C for 24 h.

2.5. Osmotic potential

The osmotic potential (Ψ_o) of primary branches (n = 6) was determined after the 30 days of the treatments, using psychrometric technique with a Vapor Pressure Osmometer (5600 Vapro, Wescor,

Logan, USA).

2.6. Measurement of gas exchange

Gas exchange measurements were taken on random primary branches using an infrared gas analyser in an open system (LI-6400, LI-COR Inc., Neb., USA) after 30 days of treatment ($n = 7$). Net photosynthetic rate (A_N), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were determined. With following conditions: a photosynthetic photon flux density (PPFD) $1000 \mu mol m^{-2} s^{-1}$ (with 15% blue light to maximize stomatal aperture), vapour pressure deficit of 2.0–3.0 kPa and air temperature of $25 \pm 2^\circ C$. The ambient CO_2 concentration (C_a) was of 400 ppm air or 700 ppm air depending on the concentration that plants were exposed to during the experiment.

Photosynthetic area was approximated as half the area of the cylindrical branches, as only the upper half received the unilateral illumination in the leaf chamber (Redondo-Gómez et al., 2010). Intrinsic water use efficiency ($iWUE$) was also calculated as the ratio between A_N and g_s . In addition, ETR_{max}/A_N ratio was calculated with the values obtained from fluorescence rapid light curves (see below) and gas exchange measurements.

Furthermore, mesophyll conductance (g_m) and maximum carboxylation rate allowed by ribulose-1,5-biphosphate (RuBP) carboxylase/oxygenase ($V_{c,max}$) were obtained by the curve-fitting method (Ethier and Livingston, 2004) using the software package developed by Sharkey et al. (2007). To attain this approach, we performed four A_N/C_i curves on random primary branches at the same environmental conditions used previously for instantaneous gas exchange measurements. An infrared gas analyser in an open system (LI-6400, LI-COR Inc., Neb., USA) equipped with a light leaf chamber (LI-6400-02B, Li-Cor Inc.) was used. When the steady-state was reached the curves were performed by decreasing C_a stepwise until $50 \mu mol mol^{-1}$. Therefore, to complete the curve the chamber conditions were restored to the initial C_a and increased stepwise until 2000 ppm (Flexas et al., 2009; Pons et al., 2009). For each curves, 12 different C_a values were used. At each step, gas exchange was allowed to equilibrate for less than 120 s to reduce changes in Rubisco activity (Long and Bernacchi, 2003).

On the same branches dark respiration rate (R_d , $\mu mol CO_2 m^{-2} s^{-1}$) measurements were performed after curves assessment as CO_2 efflux. Branches chamber was darkened for 30 min to avoid transient post-illumination bursts of CO_2 releasing (Flexas et al., 2013). Leakage of CO_2 in and out of the leaf chamber was determined with photosynthetically inactive primary branches and corrected in all curves (Flexas et al.,

2013).

2.7. Carbon and nitrogen content and stable isotope analysis

At the end of the experiment, carbon isotopic composition of the pulverized dry branches samples randomly collected ($n = 5$) was determined according to Duarte et al. (2014), using a Flash EA 1112 Series elemental analyser coupled on line via Finningan conFlo III interface to a Thermo delta V S mass spectrometer. Using the formula: $\delta^{13}C$ or $\delta^{15}N = [(R_{sample}/R_{standard})^{-1}] \cdot 10^3$, where R is $^{13}C/^{12}C$ or $^{15}N/^{14}N$, the carbon and nitrogen isotope ratio were calculated. They were expressed in delta (δ) notation, explained as the parts per thousand (‰) deviation from a standard material (PDB limestone for $\delta^{13}C$ in air for $\delta^{15}N$). The analytical precision for the measurement was 0.2‰. Carbon and nitrogen contents (%) were assessed simultaneously using the same method.

2.8. Measurement of chlorophyll fluorescence

Chlorophyll fluorescence measurements were performed using a FluorPen FP100 (Photo System Instruments, Czech Republic) in the same branches of gas exchange analysis at the end of the experiment ($n = 7$). Branches were dark-adapted for 30 min with special pliers designed for that purpose before the measurement. All the parameters were measured again in light adapted branches. As Schreiber et al. (1986) described, light energy yields of Photosystem II (PSII) reaction centers were determined with a saturation pulse method. To estimate the maximum fluorescence signal across time, a saturating light pulse of 0.8 s with an intensity of $8000 \mu mol m^{-2} s^{-1}$ was used. A comparison of the minimum fluorescence (F_0), the maximum fluorescence (F_m) and the operational photochemical efficiency values were made with the values of light and dark adapted branches. Quantum yield of PS II (QY) and relative Quantum yield of PS II (Q'Y) were calculated as F_v/F_m and Φ_{PSII} respectively. Following (Duarte et al., 2015a), maximum electron transport rate (ETR_{max}) and the chlorophyll a fast kinetics, or JIP-test (or Kautsky curves), which depicts the rate of reduction kinetics of various components of PSII, were also measured in dark-adapted leaves ($n = 5$ for each one) according to Duarte et al. (2015a), using the pre-programmed OJIP protocols of the FluorPen. All derived parameters for both RLC and OJIP were calculated according to Marshall et al. (2000) and Strasser et al. (2004) respectively (Table 1).

Table 1
Summary of fluorometric analysis parameters and their description.

Photosystem II Efficiency	
F_v/F_m	Maximum quantum efficiency of PSII photochemistry
PS II Operational and Maximum Quantum Yield (Φ_{PSII})	Light and dark-adapted quantum yield of primary photochemistry, equal to the efficiency by which a PS II trapped photon will reduce Q_A to Q_A^-
Rapid Light Curves (RLCs)	
ETR_{max}	Maximum ETR after which photo-inhibition can be observed
Energy Fluxes (Kautsky curves)	
Area	Corresponds to the oxidized quinone pool size available for reduction and is a function of the area above the Kautsky plot
W_K	Amplitude of the K-step.
Φ_{EO}	Probability that an absorbed photon will move an electron into the electronic transport chain
Φ_{R_0}	Electron movement efficiency from the reduced intersystem electron acceptors to the PSI and electron acceptors.
N	Reaction centre turnover rate
S_m	Relative pool size of PQ.
M_0	Net rate of PS II RC closure.
ABS/CS	Absorbed energy flux per leaf cross-section.
TR/CS	Trapped energy flux per leaf cross-section
ET/CS	Electron transport energy flux per leaf cross-section
DI/CS	Dissipated energy flux per leaf cross-section.
RC/CS	Number of available reaction centres per leaf cross section
Grouping Probability (P_G)	The grouping probability is a direct measure of the connectivity between the two PS II units (Strasser and Stribet, 2001)

2.9. Gauss peak-spectra pigment analysis

At the end of the experimental period, branches samples were randomly collected ($n = 5$), flash-frozen in liquid N_2 and freeze-dried for 48 h in the dark to avoid photodegradation processes (Duarte et al., 2015a). Then, samples were subsequently grinded in pure acetone and pigments extracted at -20°C during 24 h in the dark to prevent its degradation, centrifuged at 4000 rpm during 15 min at 4°C and the resulting supernatant scanned in a dual beam spectrophotometer (Hitachi Ltd., Japan) from 350 to 750 nm at 1 nm step. The resulting absorbance spectrum was used for pigment quantification by introducing the absorbance spectrum in a Gauss-Peak Spectra (GPS) fitting library, using SigmaPlot Software (Küpper et al., 2007).

2.10. Anti-oxidant enzymatic activity

Enzyme extraction was done following the methodology used by Duarte et al. (2015a). At the end of experiment, 500 mg of fresh branches samples were grounded in 8 ml of 50 mM sodium phosphate buffer (pH 7.6) with 0.1 mM Na-EDTA and were centrifuged at $10,000 \times g$ for 20 min at 4°C to obtained the soluble proteins. Three samples per treatment were used and three measurements per sample were registered. Protein content in the extracts was obtained according to Bradford (1976) protocol, using bovine serum albumin as a standard.

To quantify catalase (CAT) activity, the decrease in absorbance at 240 nm due to the consumption of H_2O_2 was measured according to Teranishi et al. (1974). The reaction mixture contained 50 mM of sodium phosphate buffer (pH 7.6), 0.1 mM of Na-EDTA, and 100 mM of

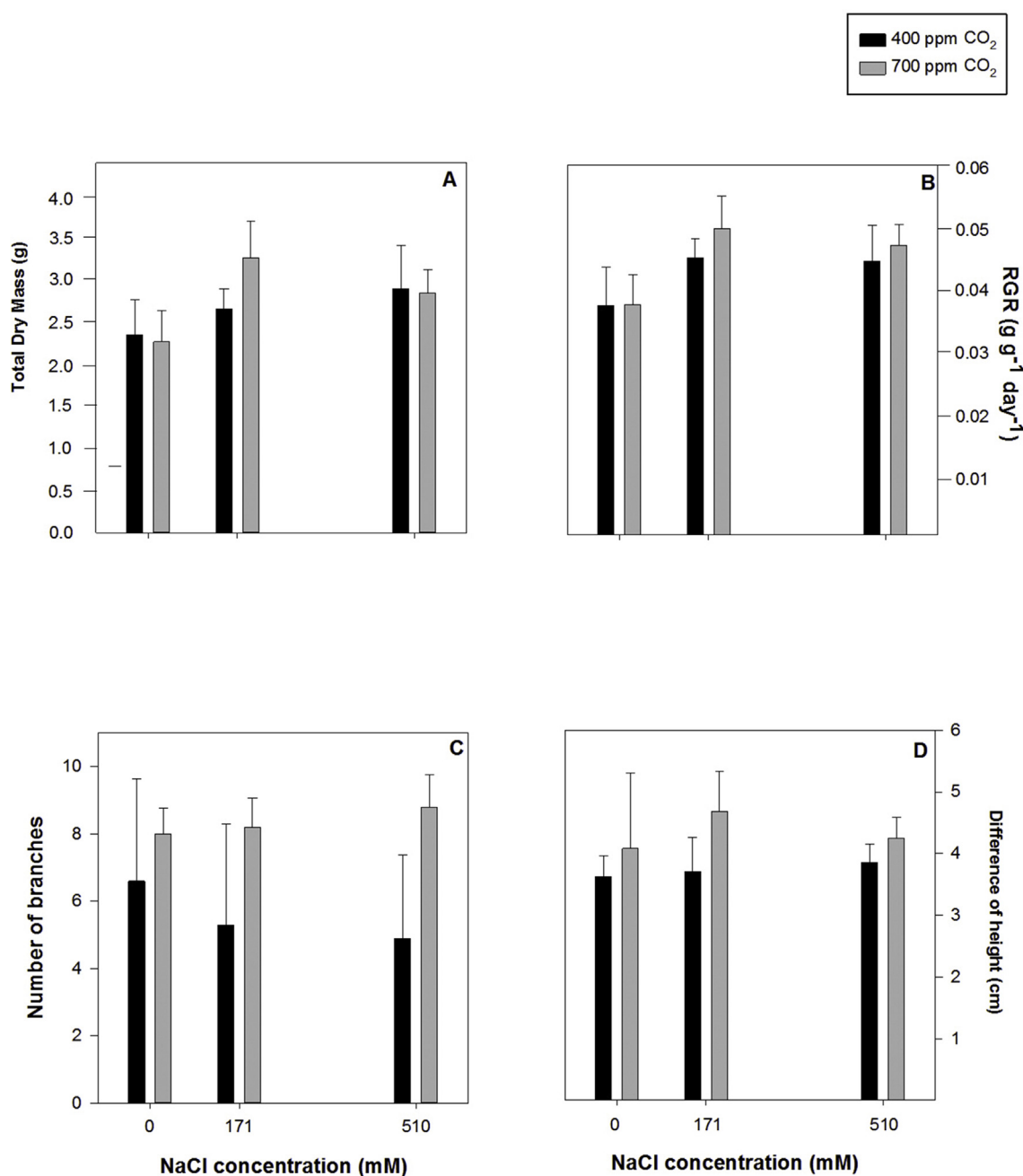


Fig. 1. Growth of *Salicornia ramosissima* in response to treatment with a range of NaCl concentrations at 400 and 700 ppm CO_2 for 30d. Relative growth rate (RGR) (A), height differences (B), ramification difference (C) and total dry mass (D). Values represent mean $\pm$ SE, $n = 10$. Different letters indicate means that are significantly different from each other (LSD test, $P < 0.05$).

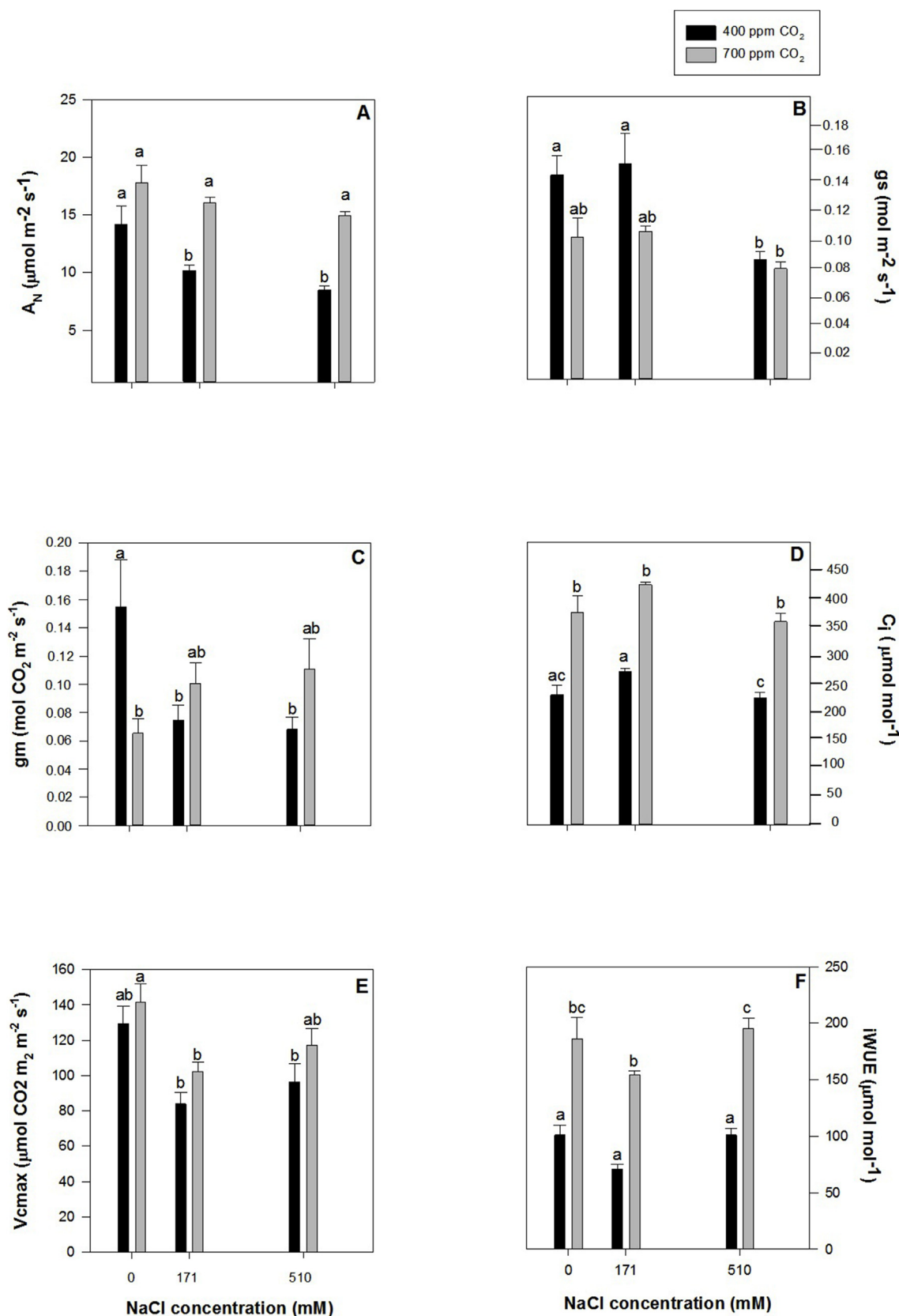


Fig. 2. Net photosynthetic rate, A_N (A), stomatal conductance, g_s (B) mesophyll conductance, g_m (C), intercellular CO₂ concentration, C_i (D), maximum carboxylation rate, V_{cmax} (E) and intrinsic water use efficiency (iWUE) (F) in randomly selected, primary branches of *Salicornia ramosissima* in response to treatment with a range of NaCl concentrations at 400 and 700 ppm CO₂ after 30d of treatment. Values represent mean $\pm$ SE, n = 4. Different letters indicate means that are significantly different from each other (LSD, P < 0.05).

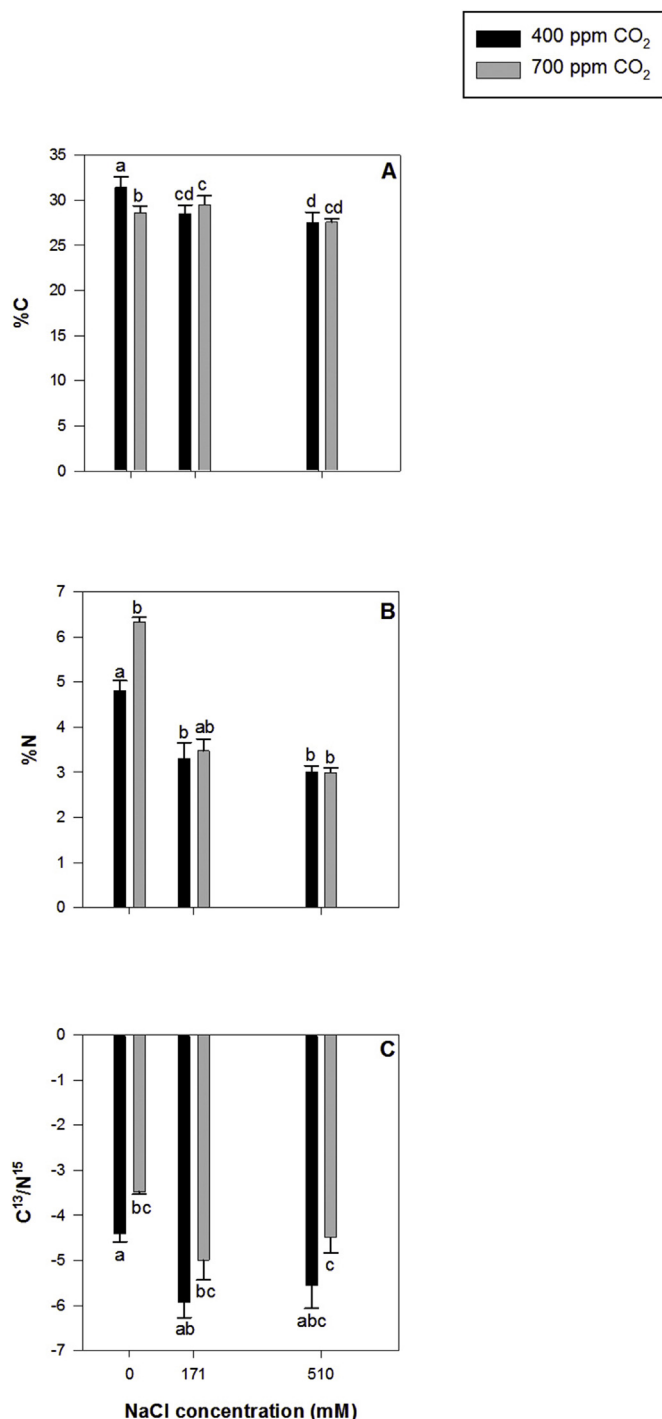


Fig. 3. Carbon percentage in leaves, %C (A), nitrogen percentage in leaves, %N (B) and carbon to nitrogen concentrations ratio, C/N (C) in randomly selected, primary branches of *Salicornia ramosissima* in response to treatment with a range of NaCl concentrations at 400 and 700 ppm CO₂ after 30d of treatment. Values represent mean ± SE, n = 5. Different letters indicate means that are significantly different from each other (LSD, P < 0.05).

H₂O₂. Ascorbate peroxidase (APX) activity was measured by monitoring the decrease in the absorbance at 290 nm. The reaction mixture contained 50 mM of sodium phosphate buffer (pH 7.0), 12 mM of H₂O₂, 0.25 mM l-ascorbate (Tiryakioglu et al., 2006). Molar coefficient of 2.8 mM⁻¹ cm⁻¹ was used to calculate the amount of ascorbate oxidized. For all this enzymatic enzymes 100 μL of vegetal extract was added at the reaction mixture to begin with the reaction. Superoxide

dismutase (SOD) activity was assayed according to Marklund and Marklund (1974) by monitoring the reduction of pyrogallol at 325 nm. The reaction mixture was 50 mM of sodium phosphate buffer (pH 7.6), 0.1 mM of Na-EDTA, 3 mM of pyrogallol, Milli-Q water. The reaction was started with the addition of 10 μL of enzyme extract. The auto-oxidation of the substrates was evaluated by control samples without the enzyme extract.

2.11. Statistical analysis

All the statistic tests were performed by a statistical software package R. The differential effect of NaCl and atmospheric CO₂ concentrations treatments were determined by Two-way analysis of variance. Multiple comparisons were analyzed by a Tukey test. Before statistical analysis, Kolmogorov-Smirnov and Levene tests were used to verify the assumptions of normality and homogeneity of variances, respectively.

3. Results

3.1. Growth analysis

Salinity and atmospheric CO₂ concentration had not any significant effect on the growth of *S. ramosissima* after 30 day of treatments (Two-way ANOVA, p > 0.05). Thus total dry mass and RGR values did not significantly vary between each specific treatment, although overall plants grown at 700 ppm of CO₂ showed higher values than their non-CO₂ supplied counterparts (Fig. 1A and B). In addition, there were no statistical differences in the height and the number of branches (Fig. 1C and D).

3.2. Plant water state

The RWC values did not show any significant differences between salinity or CO₂ treatments (Two-way ANOVA, p > 0.05), ranging between 83 and 89% for all treatments. Contrary, in Ψ_0 we found an increased with the raise of NaCl concentration in similar degree for both CO₂ levels (Two-way ANOVA: salinity, p > 0.05), with values c. -2.4, -2.7 and -4.2 MPa for 0, 171 and 510 mM NaCl, respectively (data non-presented).

3.3. Gas exchange measurements

There was a significant effect of salinity and atmospheric CO₂ concentration on the gas exchange parameters of *S. ramosissima* after 30 day of treatments (Two-way ANOVA: salinity x CO₂, p > 0.05). Thus, A_N values decreased considerably in presence of NaCl in plants grown at ambient atmospheric CO₂ concentration, while it did not vary with salinity increment at elevated level of CO₂ (Fig. 2A). g_s values decreased considerably in plants grown at the highest salinity concentration compared with those exposed to low and middle NaCl concentrations and at ambient atmospheric CO₂. The values from g_s were a 31% lower in plants grown at 700 ppm compared with those exposed to 400 ppm at 0 and 171 mM NaCl, while it did not vary between both CO₂ levels at the highest NaCl concentration (Fig. 2B).

On the other hand, g_m values decreased considerably in presence of NaCl in plants grown at 400 ppm CO₂. In addition, g_m values were lower at elevated CO₂ and absence of NaCl addition, while it increased under saline conditions (Fig. 2C). V_{c,max} decreased c. 22.5% for all treatments in presence of NaCl in the solution compared with those without NaCl supplementation (Fig. 2E). However, it was higher in plants grown at 700 ppm CO₂. Furthermore, C_i values were significantly greater in plants grown at 700 ppm for all salinity concentrations (Fig. 2D). Finally, overall iWUE did not vary between NaCl treatments, although it was considerably greater under elevated atmospheric CO₂ concentration (Fig. 2F).

3.4. Carbon and nitrogen content and stable isotope analysis

Carbon content did not highly vary between salinity and CO₂ treatments, showing in all cases values c. 29% (Fig. 3A). While N content decreased significantly with the presence of NaCl in the grown medium in similar degree for both atmospheric CO₂ levels, and it was higher in plants grown at 700 ppm in absence of NaCl addition (Two-way ANOVA: salinity x CO₂, $p > 0.05$; Fig. 3B). On the other hand, C¹³/N¹⁵ ratio increased with the presence of NaCl in the solution and were higher for the treatments grown at 400 ppm CO₂ (Two-way ANOVA: salinity x CO₂, $p > 0.05$; Fig. 3C).

3.5. Fluorescence measurements

F_v/F_m and Φ_{PSII} did not show a clear pattern of variation concerning to CO₂ and salinity treatments at the end of the experiment, showing closely similar values in all experimental treatments (Two-way ANOVA: salinity x CO₂, $p > 0.05$; Fig. 4A and B). Similarly, ETR_{max} values did not vary between treatments, showing mean values c. 40. However, the ETR_{max}/A_N remained constant for plants grown at 700 ppm CO₂. While it increased with NaCl concentration augmentation in those grown at 400 ppm CO₂ concentration (Fig. 4C).

Focusing on derived-parameters from the Kautsky curves, there is to notice that overall there was certain significant effect of CO₂ and NaCl concentrations in several variables (Two-way ANOVA: salinity x CO₂, $p > 0.05$). Thus, W_k , M_0 , N , Sm , P_G , and ϕR_0 values were lower and EOCs higher in presence of NaCl in the grown medium, and overall these values did not differ between both atmospheric CO₂ concentration treatments (Fig. 5A and B,C,E,F). Contrary Area was not significant influenced by NaCl concentration, but was 35% greater in plants grown at 700 ppm and 510 mM NaCl compared those expose to 400 ppm CO₂ (Two-way ANOVA: CO₂, $p < 0.05$; Fig. 5D). On the other hand, the energetic fluxes on a leaf cross-section basis (phenomological fluxes) showed that ABS/CS , TR/CS , ET/CS and DI/CS did not present any statistical relationship with NaCl concentration, but these values were significantly higher in plants grown at 700 ppm CO₂ at the highest salinity concentration treatment (Two-way ANOVA: CO₂, $p < 0.05$; Fig. 6A–D).

3.6. Pigment concentration

Pigments concentration analysis showed that chlorophyll *a* and *b* concentrations did not vary between the different NaCl treatments, but overall these values were significantly higher at 700 ppm than at 400 ppm CO₂ (Two-way ANOVA: CO₂, $p < 0.05$; Table 2). Being the differences more accused at 0 and 171 mM NaCl. A similar trend could be seen in neoxanthin values, while violaxanthin and zeaxanthin concentration only varied between CO₂ levels at 0 mM NaCl.

3.7. Anti-oxidant enzymatic activity

CAT enzyme activity did not show a clear trend regarding salinity and CO₂ increment (Fig. 7A). While we found that APx and SOD enzymes activities were higher in plants grown at 700 ppm at 171 and 510 mM NaCl, and decreased in plant exposed to 0 mM NaCl compared with those plants grown at 400 ppm CO₂ (Two-way ANOVA: salinity x CO₂, $p > 0.05$; Fig. 7B and C).

4. Discussion

This study indicated that atmospheric CO₂ enrichment improved the physiological performance of *S. ramosissima* under suboptimal salinity concentration (NaCl excess), which becomes relevant for the future development of this interesting multifunctional cash crop in a context of future global climate change scenario. According to these results, Geissler et al. (2009a,b; 2015) found that the rising atmospheric CO₂

concentration had an ameliorative effect on salinity stress experienced by the halophytes C₃ *Aster trifolium* and C₄ *Atriplex nummularia*, but without a positive effect on the biomass production. Accordingly, we found that *S. ramosissima* biomass did not highly vary with the atmospheric CO₂ increment, which could be linked with the investment of certain amount of the energy fixed during the photosynthetic process in several mechanisms for defense against salinity excess, as was

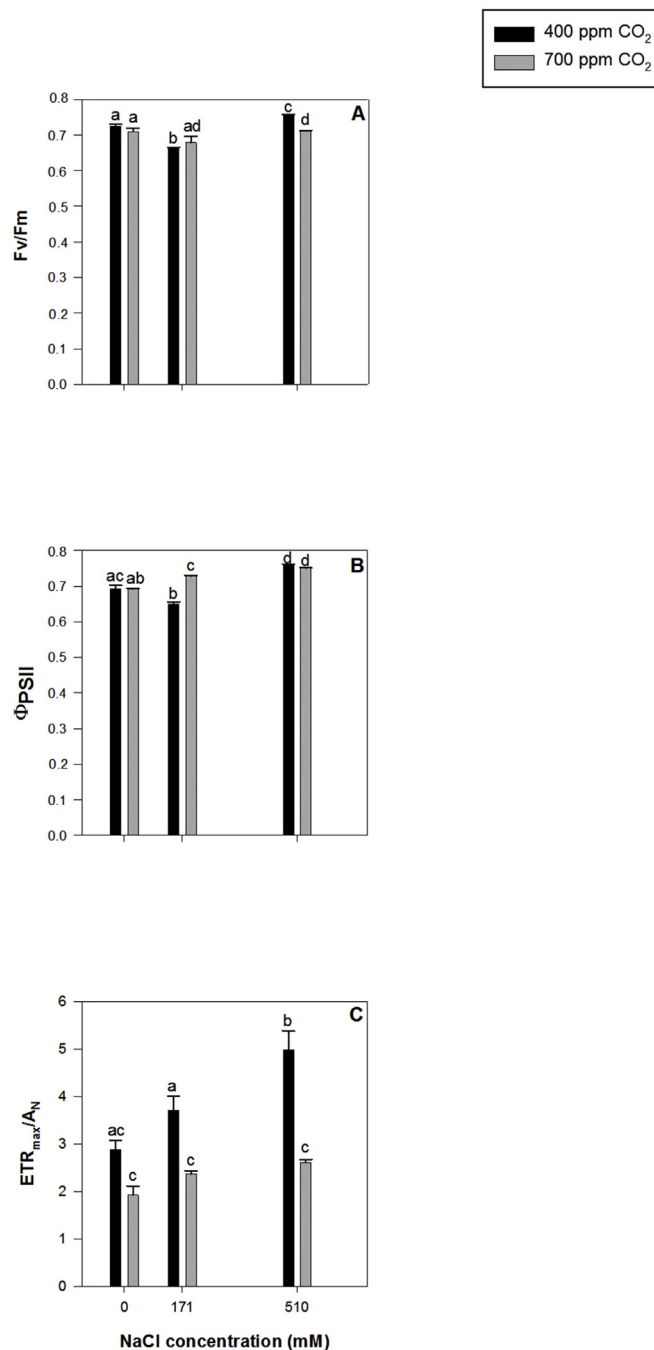


Fig. 4. Maximum quantum efficiency of PSII photochemistry, F_v/F_m (A), Light and dark-adapted quantum yield of primary photochemistry, equal to the efficiency by which a PS II trapped photon will reduce Q_A to Q_A^- , Φ_{PSII} (B) and Maximum ETR after which photo-inhibition can be observed net photosynthetic rate ratio, ETR_{max}/A_N (C) in dark adapted randomly selected, primary branches of *Salicornia ramosissima* in response to treatment with a range of NaCl concentrations at 400 and 700 ppm CO₂ after 30d of treatment. Values represent mean $\pm$ SE, $n = 5$. Different letters indicate means that are significantly different from each other (LSD, $P < 0.05$).

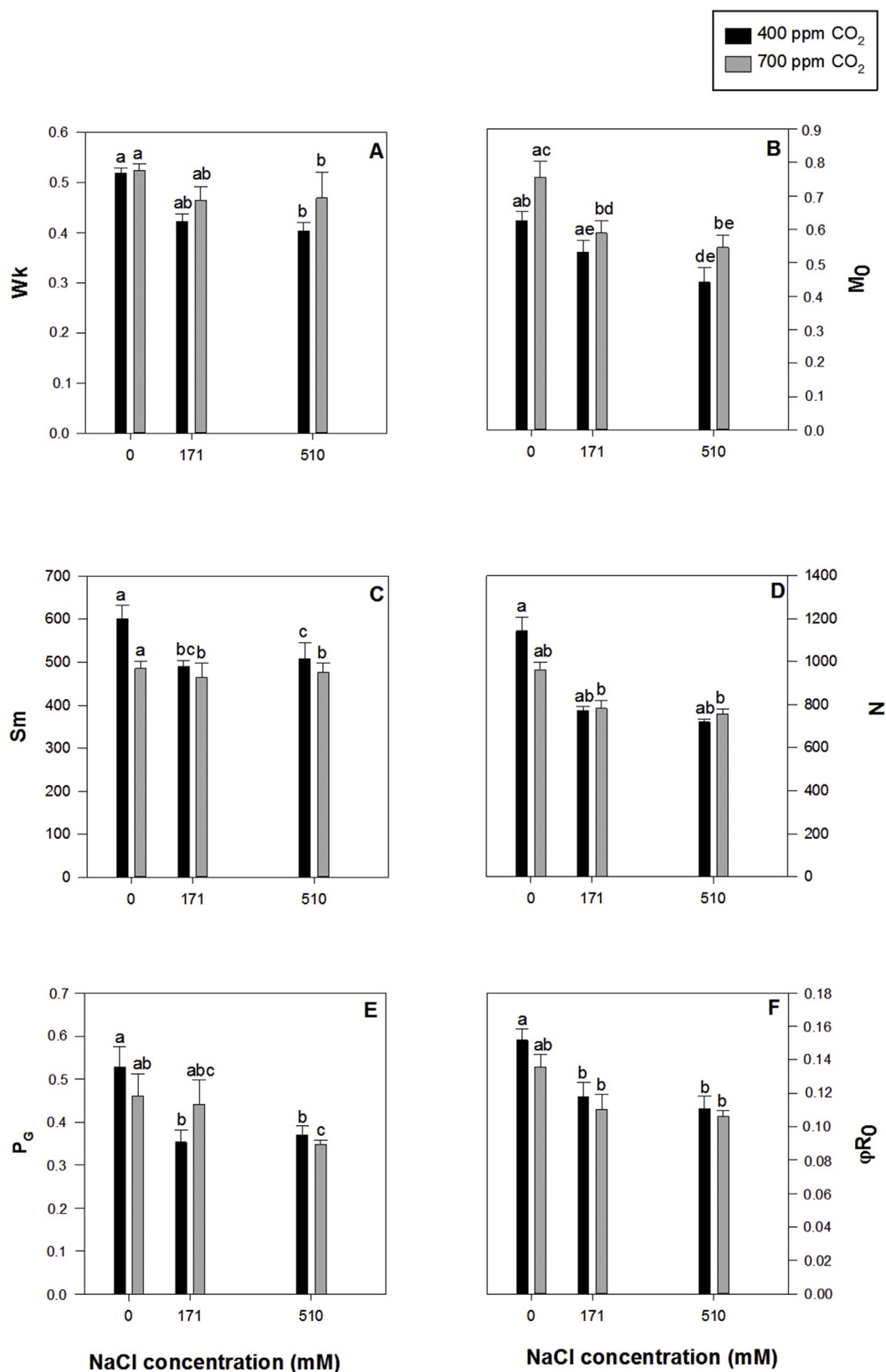


Fig. 5. Amplitude of the K-step, Wk (A), net rate of PS II RC closure, M₀ (B), relative PQ pool size, Sm (C), reaction centre turnover rate, N (D), grouping probability, PG (E) and maximum yield of primary photochemistry, ϕR_0 , (F) in dark adapted randomly selected, primary branches of *Salicornia ramossissima* in response to treatment with a range of NaCl concentrations at 400 and 700 ppm CO₂ after 30d of treatment. Values represent mean $\pm$ SE, n = 5. Different letters indicate means that are significantly different from each other (LSD, P < 0.05).

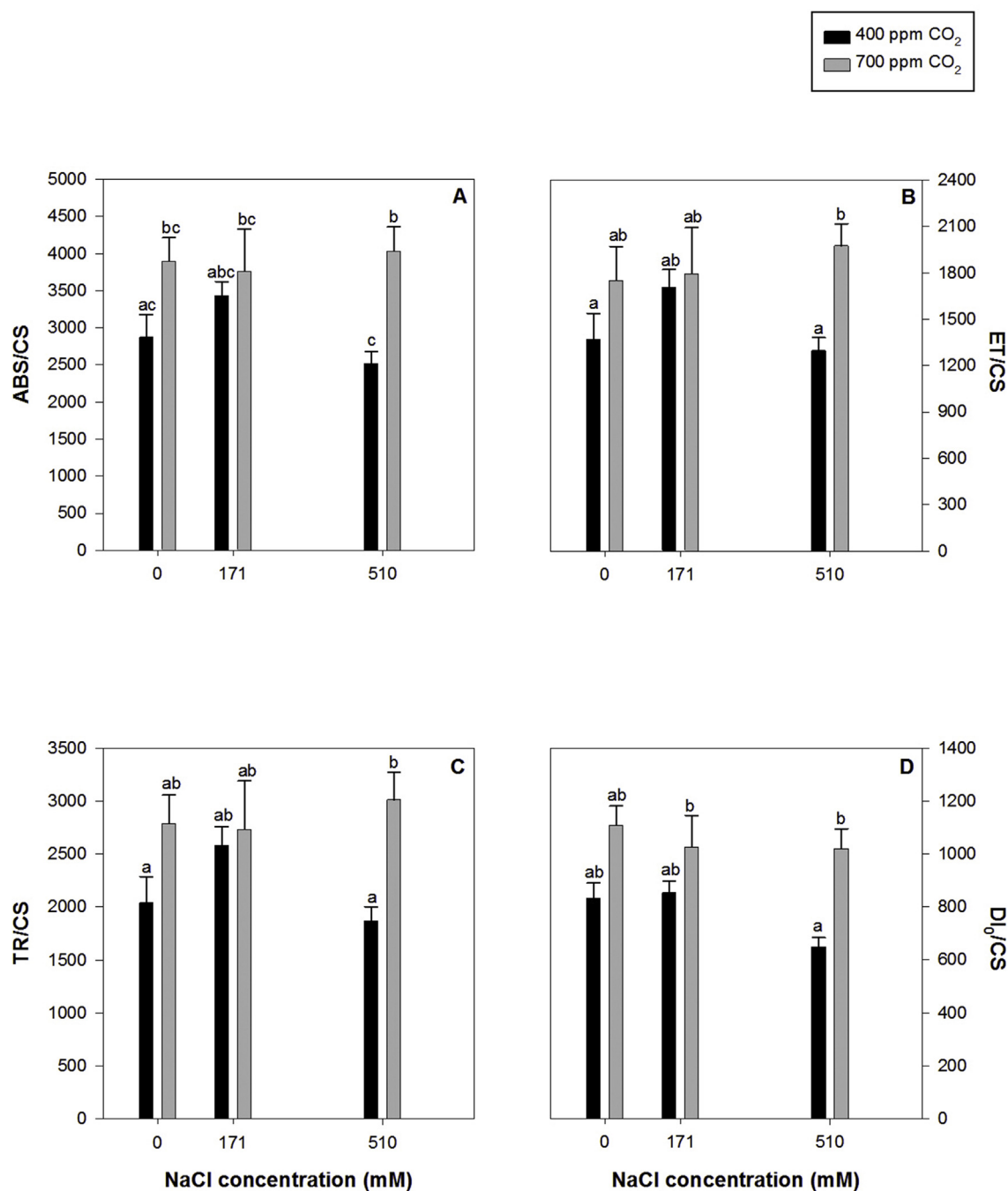


Fig. 6. Number of available reaction centres, ABS/CS (A), trapped energy flux, ET₀/CS (B) electron transport energy flux TR₀/CS (C) and dissipated energy fluxes, DI₀/CS (D) per leaf cross section and the performance index on absorption basis, PI (F) in dark adapted randomly selected, primary branches of *Salicornia ramosissima* in response to treatment with a range of NaCl concentrations at 400 and 700 ppm CO₂ after 30d of treatment. Values represent mean $\pm$ SE, n = 5. Different letters indicate means that are significantly different from each other (LSD, P < 0.05).

Table 2

Photosynthetic pigments concentrations ($\mu\text{g/g}$) and DES state in randomly selected branch of *Salicornia ramosissima* in response to treatment with a range of NaCl concentrations at 400 and 700 ppm CO₂ after 30d of treatment. Values represent mean $\pm$ SE, n = 5. Different letters indicate means that are significantly different from each other (LSD, P < 0.05).

[NaCl] (mM)	[CO ₂] (ppm)	Chl a	Chl b	Phe a	b-carotene	Lutein	Neoxanthin	Violaxanthin	Zeaxanthin	DES
0	700	247.2 $\pm$ 15.2 ^{ab}	91.5 $\pm$ 6.7 ^{ab}	13.4 $\pm$ 2.3 ^a	7.5 $\pm$ 0.4 ^a	22.4 $\pm$ 2.3 ^a	8.9 $\pm$ 0.6 ^a	8.6 $\pm$ 1.1 ^a	8.7 $\pm$ 1.6 ^a	0.50 $\pm$ 0.03 ^a
	400	205.9 $\pm$ 16.7 ^a	82.6 $\pm$ 5.1 ^a	16.0 $\pm$ 2.5 ^a	6.2 $\pm$ 0.3 ^a	17.2 $\pm$ 2.4 ^b	4.8 $\pm$ 1.2 ^b	7.1 $\pm$ 1.6 ^a	7.9 $\pm$ 1.6 ^{ab}	0.45 $\pm$ 0.11 ^b
171	700	161.8 $\pm$ 6.5 ^c	51.4 $\pm$ 1.9 ^c	5.3 $\pm$ 0.2 ^b	5.1 $\pm$ 0.3 ^a	13.6 $\pm$ 0.8 ^b	5.6 $\pm$ 0.4 ^b	5.7 $\pm$ 0.3 ^a	4.8 $\pm$ 0.3 ^b	0.54 $\pm$ 0.01 ^{ab}
	400	121.8 $\pm$ 16.2 ^{bc}	44.4 $\pm$ 1.6 ^c	8.7 $\pm$ 2.5 ^{ab}	7.3 $\pm$ 1.2 ^a	13.1 $\pm$ 2.6 ^b	4.0 $\pm$ 0.8 ^b	7.9 $\pm$ 1.8 ^a	5.9 $\pm$ 1.2 ^{ab}	0.54 $\pm$ 0.02 ^{ab}
510	700	166.6 $\pm$ 8.8 ^{bc}	54.0 $\pm$ 3.1 ^{bc}	4.4 $\pm$ 0.5 ^b	5.3 $\pm$ 0.3 ^a	13.4 $\pm$ 0.8 ^b	5.8 $\pm$ 0.4 ^b	5.7 $\pm$ 0.7 ^a	5.1 $\pm$ 0.3 ^b	0.52 $\pm$ 0.02 ^{ab}
	400	166.1 $\pm$ 7.5 ^{bc}	58.3 $\pm$ 4.5 ^{bc}	7.0 $\pm$ 1.5 ^{ab}	5.5 $\pm$ 0.3 ^a	14.2 $\pm$ 0.9 ^b	4.9 $\pm$ 0.4 ^b	6.8 $\pm$ 0.8 ^a	5.5 $\pm$ 0.4 ^b	0.56 $\pm$ 0.02 ^{ab}

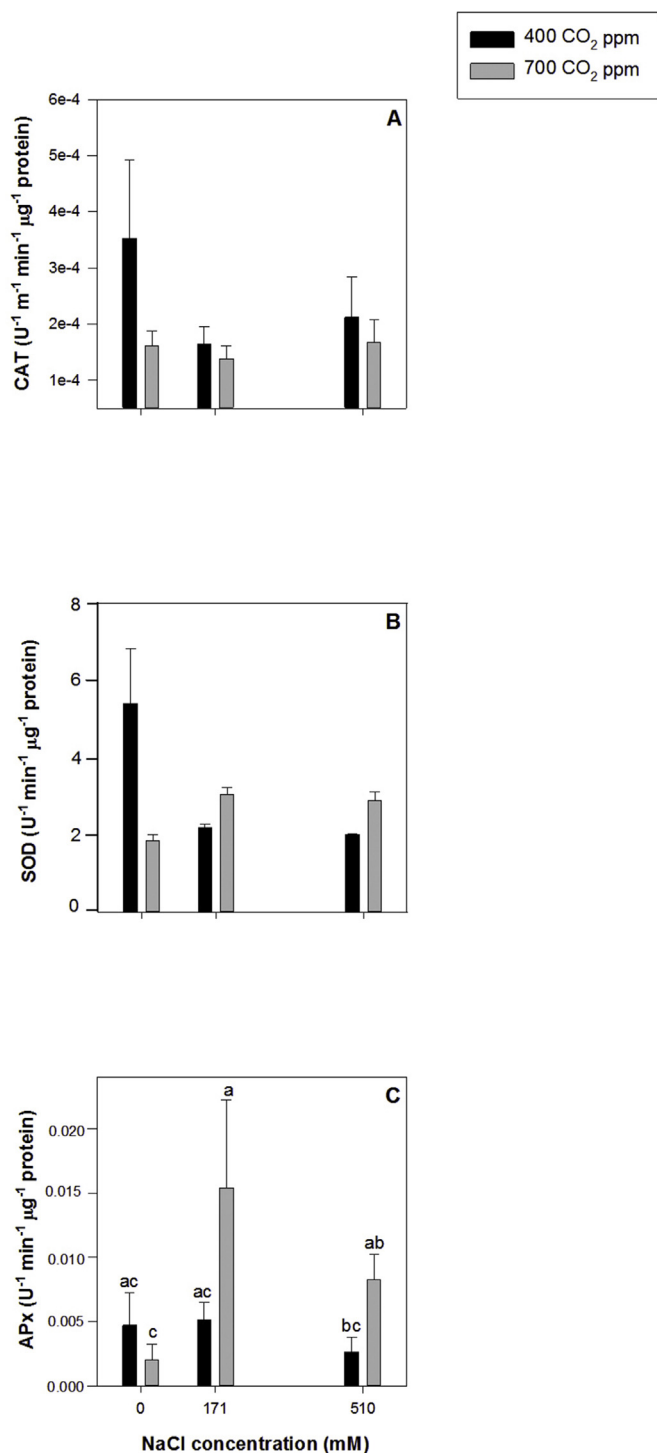


Fig. 7. Catalase (CAT) (A), and superoxide dismutase (SOD) (B), ascorbate peroxidase (APx) (C) enzymatic activities in randomly selected, primary branches of *Salicornia ramosissima* in response to treatment with a range of NaCl concentrations at 400 and 700 ppm CO₂ after 30d of treatment. Values represent mean $\pm$ SE, $n = 3$. Different letters indicate means that are significantly different from each other (LSD, $P < 0.05$).

previously suggested for other halophytes (Geissler et al., 2015).

Among physiological processes, photosynthetic metabolism has demonstrated to be especially sensible to salt excess (Flexas et al., 2004). Thus, it has been described that salinity affects photosynthetic metabolism mainly in terms of diffusion of CO₂ in leaves through a decrease in stomatal and mesophyll conductance (Flexas et al., 2004;

Fanourakis et al., 2015). This could lead to a reduction in C_i with the consequence decrease in A_N (Verena et al., 2016). In this regard, our study indicated that at elevated salinity and ambient atmospheric CO₂ level photosynthetic responses of *S. ramosissima* were clearly impaired, as indicated the reduction of several parameters. Thus, A_N values decreased with NaCl supplementation, together with a sharply decrease in g_m for both salinity treatments and reduction in g_s only in the highest salinity concentration. However, although overall salinity involved a simultaneous reduction in A_N , g_m and g_s , there would not have a clear direct relation between them, since contrary to be expected C_i values did not change with increasing NaCl concentration. A decline in both CO₂ assimilation (A_N) and diffusion through leaf (i.e. g_s and g_m) without a corresponding reduction in C_i may be interpreted as a direct effect of NaCl on the carbon fixation apparatus (Brugnoli and Lauteri, 1991). Thereby, salinity effects are related to alterations in metabolic functions such as protein synthesis, which determine photoinhibition levels (Ohad et al., 1984), and they may lead to biochemical limitations that affect carboxylase activity of RuBisCO (Antolín and Sánchez-Díaz, 1993). Therefore, the decrease of A_N could be linked by alteration of Rubisco activity, as it has been previously described for other halophytes, such as *S. densiflora* in response to NaCl stress (Mateos-Naranjo et al., 2015). Hence, we found that $V_{c,max}$ values decreased in plants grown in presence of NaCl addition compared with those without NaCl supplementation.

Despite the evident impact of salinity excess on some of the essential steps of *S. ramosissima* photosynthetic pathway, we found that rising atmospheric CO₂ had a positive effect on its photosynthetic apparatus performance. A stimulation of photosynthesis in C_3 species is a general response to CO₂ atmospheric concentration enrichment (Duarte et al., 2014; Geissler et al., 2015), varying between species and environmental conditions (Hussini et al., 2017). The results of this experiment were in concordance with that trend, since at 700 ppm CO₂ A_N values remained at the same level as the non-salinized plants. Furthermore, g_s values were considerably lower at the higher atmospheric CO₂ concentration and it was not affected by salinity increment; being this reduction explained by the increase of C_i originated by the elevated CO₂ concentration, which could promote partial stomatal closure, although the mechanism whereby stomata respond to the CO₂ signal remains unknown (Robredo et al., 2007). However, there is to notice that g_s reduction would contribute to preserve the trade-off between CO₂ acquisition for photosynthetic process and water losses in *S. ramosissima* plants. Hormaetxe et al. (2006) indicated that an increment in Ψ_o together with a decrease in g_s , as was also reported in our study, would contribute to a better water balance under stress, idea that was supported by the overall higher $iWUE$ values under rising atmospheric CO₂ conditions. According to our study, several authors have reported an increment of $iWUE$ in enrichment CO₂ atmosphere combined with NaCl salinity in the grown medium (Geissler et al., 2009a; b; Mateos-Naranjo et al., 2010b; Reef et al., 2015). Furthermore, the beneficial effect of CO₂ enrichment in A_N could be ascribed to the reduction on mesophyll CO₂ diffusion and biochemical limitations imposed by salinity stress aforementioned. So we found that overall maximum $V_{c,max}$ and g_m values of *S. ramosissima* tended to increase with atmospheric CO₂ concentration under saline conditions; so we could hypothesized that the resistance to CO₂ diffusion imposed by the stomatal resistance under saline conditions (as indicated the lower g_s values), could be in certain degree counterbalance through reducing mesophyll and biochemical limitations, as indicated the maintenance of RuBisCO carboxylation and g_m in values close to control plants. In fact, Centritto et al. (2003) indicated that the main limitation to CO₂ diffusion in presence of salt NaCl stress was caused by a reduction in the mesophyll conductance, rather than stomatal effects. Furthermore, the lower C/N and C^{13}/N^{15} ratios recorded in *S. ramosissima* under elevated CO₂ supported our hypothesis. These lower values were related with an increment in N acquisition and could be linked with the maintenance of RuBisCO functions. Since the activity of the Rubisco enzyme is in

relation with the photosynthetic capacity along with N content, as great amounts of N are supplied in Rubisco protein (Evans, 1989; Makino et al., 1992).

On the other hand, the ameliorative effect of rising atmospheric CO₂ concentration on the deleterious impacts of NaCl excess on the photosynthetic apparatus of *S. ramosissima* was ascribed by variations of PSII photochemistry, which would improve its functionality in terms of energy harvesting, transport and dissipation. It has been stated that salinity excess could impair PSII photochemistry in different ways; thus several studies have indicated that the electron transport chain efficiency decrease along with the ability to use the incident photons (Panda et al., 2006; Mateos-Naranjo et al., 2007; Duarte et al., 2015b). Causing a decrease in the maximum yield for primary photochemistry (Kalaji et al., 2011). Also, Duarte et al. (2015b) indicated that salinity excess contributed to decline the reaction centers available for photon harvesting in the halophyte *Spartina patens*. However, our fluorescence data showed that the reduction of N and the ϕ_{R0} in presence of salt was in certain degree attenuated by the high CO₂ concentration. In addition, M_0 , ABS/CS, TR_0 /CS and ET_0 /CS values were higher for those plants, indicating that energy harvesting and the electron flux were more efficient and active in presence of salt when *S. ramosissima* plants were exposed to 700 ppm CO₂, despite of the higher P_G values showed at ambient CO₂ concentration. Moreover, we found that the DI_0 /CS increased at the highest salinity concentration, this trend similar to TR_0 /CS and ET_0 /CS could be explained by the higher values for ABS/CS. Being this parameter related with the activation of some defense mechanism to protect the photochemical apparatus through the dissipation of energy excess as heat (including NPQ that is correlated with the xanthophyll cycle) along with the photorespiration (Duarte et al., 2017). Thus we found several signs of the activation of this cycle, such as the greater concentration of zeaxanthin and violaxanthin at higher atmospheric CO₂ concentration and the rise observed at DES with NaCl supplementation (Duarte et al., 2015b). This cycle is activated as a consequence of an excess of light energy that decrease the lumen pH dissipating excessive energy and reducing the PSII and associated proteins excessive protonation (Duarte et al., 2015b). Therefore the positive impact of atmospheric CO₂ enrichment on photochemical apparatus of *S. ramosissima* was also linked with the enhanced of the efficiency for energy excess dissipation, which could contribute to reduce the accumulation of reactive oxygen species (ROS). Being the lower risk of ROS production supported by the decrease in ETR_{max}/A_N ratio recorded in *S. ramosissima* plants grown at elevated NaCl and CO₂ concentrations, since this ration has been considered an indicator of ROS production in plants in response to several stress (Salazar-Parra, 2012; Hussin et al., 2017). Finally another mechanism to reduce the accumulation of ROS is the modulation of the anti-oxidative stress enzymes machinery (Geissler et al., 2009a). Our results indicated that SOD enzyme activities tended to be higher in plants grown under elevated NaCl and atmospheric CO₂ concentration compared with their non-CO₂ supplied counterparts. The H₂O₂ created in presence of many types of stress due to photorespiration or the SOD activity had two ways to be reduced, through the catalase and the ascorbate peroxidase activities (Pérez-López et al., 2009). Our data showed that APx enzyme activities followed the SOD activity trend in plants grown at 700 ppm CO₂, which indicated the CO₂ increment would modulated APx enzyme activity in order to cope with salinity stress.

5. Conclusion

This study indicates that atmospheric CO₂ enrichment would increase the tolerance of the halophyte *S. ramosissima* to suboptimal salinity concentration (NaCl excess). Thus, we found that plants grown under elevated CO₂ and salt were able to maintain greater values of A_N than their non-CO₂ supplied counterparts. This positive effect was in certain degree linked with a reduction on g_m and $V_{c,max}$ limitations imposed by salinity excess. Also, these plants showed a better water

balance, as indicated the greater Ψ_w values, due to its lower values of g_s together with an overall enhance on Ψ_o with NaCl concentration increment. However the positive effect on the carbon assimilation capacity did not results to a significant biomass variation, being this lack of correlation ascribed by the investment of part of the energy fixed for salinity stress defense mechanisms, such as pigments productions or anti-oxidative stress enzymes modulation. This fact was supported by the greater values obtained of zeaxanthin and violaxanthin concentrations and SOD and APx enzymes activities, which could have led to the capacity of maintaining active the photochemical machinery even at high salinities, in terms of light energy harvesting, transport and dissipation, reducing in this way the risk of ROS production, as indicated the lower values ETR_{max}/A_N ratio values. For all these reasons, we can conclude that this multifunctional cash crop specie could be an interesting suitable natural resource to be cultivated in a future climate change scenario, where an increment of soil salinity concentration it is expected together with the atmospheric CO₂ enrichment.

Contributions

SRG, EMN and YI conceived and designed research. JAPR, YI and JMBP conducted experiments. BD and IC contributed with new analytical tools. JAPR, EMN and BD analyzed data. JAPR wrote the manuscript. All authors read and approved the manuscript.

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